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Biological control of eucalyptus bacterial wilt with rhizobacteria

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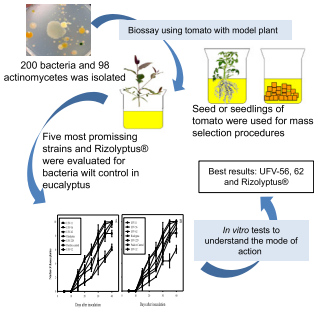
HIGHLIGHTS

- UFV-56 (*Bacillus thuringiensis*) and UFV-62 (*B. cereus*) suppressed eucalyptus wilt.
- Selection of antagonists using tomato as a model system was a successful approach.
- UFV-56 apparently reduced bacterial wilt by producing HCN and volatile compounds.
- UFV-62 apparently reduced bacterial wilt of eucalyptus by producing siderophores.

GRAPHICAL ABSTRACT



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ABSTRACT

The antagonistic potential of 298 rhizobacteria obtained from the rhizosphere and rhizoplane of tomato and eucalyptus plants was assessed for the control of bacterial wilt of eucalyptus caused by *Ralstonia solanacearum*. Several tests were performed using tomato plants as a screening system to select efficient rhizobacteria. Different methods for antagonist delivery and pathogen inoculation were evaluated: (1) seeds were microbiolized (soaked for 12 h in a suspension of the antagonist propagules) and germinated seedlings had their roots immersed in the pathogen inoculum suspension; (2) seedlings originated from microbiolized seeds were transplanted to soil infested with *R. solanacearum* and (3) roots of seedlings were immersed in a suspension of propagules of the antagonist and subsequently in a suspension of *R. solanacearum*. Nine isolates (UFV-11, 32, 40, 56, 62, 101, 170, 229, and 270) were selected as potential antagonists to *R. solanacearum* as they suppressed bacterial wilt in at least one of the methods assessed. The selected antagonists were evaluated against two isolates of *R. solanacearum* using *in vitro* and *in vivo* (inoculated eucalyptus) tests. Isolates UFV-56 (*Bacillus thuringiensis*), UFV-62 (*Bacillus cereus*) and a commercial formulation of several rhizobacteria (Rizolyptus®) suppressed bacterial wilt in eucalyptus protecting the plants during the early stages of development.

1. Introduction

Eucalyptus has become an important forest species for wood, pulp, biomass and other uses worldwide. In tropical areas, bacterial wilt caused by *Ralstonia solanacearum* (Yabuuchi et al., 1995) has caused extensive damage in plantations and is a limiting biotic factor to eucalyptus cultivation in some regions (Alfenas et al., 2006). The disease was already reported in the main producing regions of Australia (Akiew and Trevorrow, 1994), Uganda (Roux et al., 2001), South Africa (Coutinho et al., 2008), China (Wu and Liang, 1988), Taiwan (Wang, 1992), Venezuela (Ciesla et al., 1996) and Brazil (Dianese et al., 1990). Besides direct crop losses that may reach 70% (Ran et al., 2005b), there is an increase in production costs,

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mainly of those related to pathogen eradication and greenhouse structural adaptations aimed at minimizing the risks of contamination of seedlings, mini-stumps and other vegetative propagules (Alfenas et al., 2006).

Genetic and ecological characteristics of the pathogen impose difficulties to the control of bacterial wilt of eucalyptus. *R. solanacearum* is known to have high genetic variability (Hayward, 1991) and there is variation in host range, geographic distribution, pathogenicity and physiological properties. Before the genomics era, strains of *R. solanacearum* used to be classified based on the differential pathogenicity to distinct hosts (Buddenhagen et al., 1962) and also on biovars, defined as physiologic groups that vary regarding the capacity to use carbohydrates (Hayward, 1994). Recently, new groups of the pathogen have been proposed by Fegan and Prior (2005). Individuals can be grouped based on multiplex PCR, and several sequevars, based on the partial sequence of the endoglucanase gene (*egl*) are defined (Poussier et al., 2000). Currently, these properties form the basis of the most accepted classification system.

R. solanacearum race 1 is the most widespread variant of the pathogen associated with eucalyptus plants, however biovars vary according to region. In South America biovar 1 is the most commonly found, while in Asia and Australia biovar 3 is the most prevalent (Coutinho et al., 2008; Wu and Liang, 1988). Recently, Marques et al. (2012) described isolates of *R. solanacearum* race 3 biovar 2 pathogenic to eucalyptus plants in Brazil. Additionally, isolates of sequevars 37, 41 and a new sequevar of the phylotype II group were recently described in eucalyptus plants (Fonseca et al., 2013).

The wide host range and the ability of the bacteria to survive in the rhizosphere of alternative hosts, cultivated or not, or even in bare soil, favor the maintenance of epidemiologically relevant population in the soil. Additionally, *R. solanacearum* can be easily dispersed by infected seedlings, irrigation and rain water and by tools used for cultural practices (Coutinho et al., 2008; Hayward, 1994). In addition to the genetic and ecological characteristics that challenge bacterial wilt control, no chemical compounds are effective against the pathogen and crop resistance as well as cultural control measures are practically ineffective (Javier, 1994; Lopes and Takatsu, 1997).

Biological control of eucalyptus bacterial wilt mediated by rhizobacteria can be an alternative to disease management. Rhizobacteria are natural soil inhabitants capable of colonizing the root system of plants and several characteristics enable them to be used as antagonists to plant pathogens (Antoun and Kloepper, 2001). Rhizobacteria have demonstrated good colonization and survival in the rhizosphere. Furthermore, most of the bioactive products produced by the antagonists have long shelf life (Schisler et al., 2004).

Most successful attempts to control eucalyptus bacterial wilt were achieved with strains of *Pseudomonas fluorescens* characterized as a plant growth promoting rhizobacteria (PGPR). The incidence of bacterial wilt in eucalyptus seedlings treated with this biocontrol agent was reduced by 45% (Ran et al., 2005a,b). Nevertheless, despite the promising characteristics of *Pseudomonas* spp., to date, the most commonly reported group of rhizobacteria used as biocontrol agents is comprised of gram-positive bacteria, mainly by the species of *Streptomyces* and *Bacillus* (Emmert and Handelsman, 1999; Koberl et al., 2013). These bacteria can colonize the rhizosphere of plants in different habitats and they can form resistant spores which can play an important role for the development of stable formulated products.

Many studies have been conducted to explore the biocontrol capacity of these organisms, and their capacity to produce antibiotics makes them a target for the biological control of plant diseases (Raaijmakers and Mazzola, 2012; Yanes et al., 2012; Raaijmakers et al., 2002). Few studies in the world have been conducted to assess the use of rhizobacteria as biological control agent of eucalyptus bacterial wilt, none in Brazil. The main objective of this study was to obtain rhizobacteria isolates to be used as a biocontrol agent of bacterial wilt of eucalyptus seedlings. To achieve this goal we isolated rhizobacteria (actinomycetes and bacteria) from the rhizosphere and rhizoplane of tomato and eucalyptus plants and screened them regarding their capacity of promoting biocontrol of bacterial wilt using tomato plants as a model system. Finally, we assessed the efficiency of the selected rhizobacteria in reducing wilt intensity in eucalyptus seedlings and attempted to elucidate the most likely mode of antagonism involved.

2. Material and methods

2.1. Bacterial strains and growth conditions

The strain of *R. solanacearum*, phylotype II, biovar I, coded as RS 295 belongs to the *R. solanacearum* culture collection of Embrapa Hortaliças and was used in the experiments. This strain was isolated from a eucalyptus plant collected in the municipality of Carbonita, Minas Gerais state, in southeast Brazil.

To obtain the inoculum suspension of *R. solanacearum*, the strain was streaked on 523 medium (Kado and Heskett, 1970) and incubated for 48 h at 28 ± 1 °C. After incubation, saline solution (0.85% NaCl) was used to wash the colonies and the suspension was collected in a becker. The concentration of the bacterial cell suspension was adjusted to $OD_{540} = 0.2$, which corresponded to approximately 5×10^7 CFU/mL. Rhizobacteria and actinomycetes were isolated as previously described (Romeiro, 2007). Ten grams of root or soil from the eucalyptus rhizosphere were mixed with saline solution (0.85% NaCl) and kept under agitation for 24 h at 28 °C in an Erlenmeyer flask. Diluted soil samples (10^{-7} and 10^{-8}) were taken and streaked onto 523 medium in Petri plates which were kept at 28 ± 1 °C for 24 h. Individualized colonies of different color, size and shape, were transferred to test tubes containing 523 medium. The isolates were maintained in tubes containing sterilized water.

For isolation of actinomycetes, soil samples from eucalyptus and tomato rhizosphere were processed as described before, but soil suspension in saline solution were kept at 70 °C for three days (Pramer and Schmidt, 1964) before being subjected to serial dilutions and streaked onto 523 medium. The colonies that developed on 523 medium were transferred to test tubes containing soil-agar extract medium.

2.2. Mass selection

Seeds or seedlings of tomato cv. Santa Clara were used for mass selection procedures. Eucalyptus plants were not used at this stage. Tomato is highly susceptible to bacterial wilt and allows for fast plant and disease development, thus we used this plant species as a model. The experiments were conducted from January to May 2010, under greenhouse conditions, with favorable conditions for the development of the disease (maximum 30 °C and minimum 22 °C). After 2 days, the plate was flooded with saline solution (0.85% NaCl) and the suspension was adjusted to $OD_{540} = 0.2$. A mixture of 80% dystrophic red-yellow Latosol soil and 20% sand was used as non-sterilized substrate, henceforth referred to as soil mixture, in which tomato plants were grown. To investigate the potential of rhizobacteria as biocontrol agent, each antagonist candidate strain was assessed under three combinations of delivery-inoculation procedures as described below.

2.2.1. Rootlets of seedlings originated from microbiolized seeds were inoculated by immersion in a suspension of R. solanacearum (MSRI)

Previously disinfested seeds (70% ethanol/30 s and 2.5% NaClO/2 min) were immersed in saline suspension of antagonist propagules for 12 h. The concentration of the suspension of antagonist propagules was not adjusted. Seeds were put to germinate in pots containing the soil mixture and kept in the greenhouse for 45 days. After this period, plants were removed from pots, the roots were washed with running tap water and immersed in a suspension of *R. solanacearum* cells, OD_{540} = 0.2, for 1 min. Seedlings were then transplanted to pots containing 1 L of non-sterilized substrate.

2.2.2. Seedlings originated from microbiolized seeds and transplanted to artificially infested soil (MSIS)

Seedlings from microbiolized seeds were obtained as described above. Twenty-five milliliter (proportion 1:8 v/v) of inoculum suspension of *R. solanacearum*, OD_{540} = 0.2, was added to each pot containing 1 L non-sterilized substrate. Pots with infested soil mixture were kept at 30 ± 2 °C for 24 h. After that period, the seedlings were transplanted to pots.

2.2.3. Immersion of seedling rootlets in the suspension of antagonist propagules plus supplementation of antagonist suspension applied to the soil (RASAS)

Tomato seeds were seeded in 128-cell trays filled with Plantmax® (Eucatex Agro, Paulínia, SP, Brazil), a commercial substrate composed of grounded *Pinus* spp. wood chips and vermiculite, kept in greenhouse for 45 days when they were removed from the cells. Seedlings had their root system washed in running tap water and immersed in a suspension of the antagonist propagules for 1 min. Seedlings were transplanted to 30 L pots with non-sterilized substrate and 25 mL of the antagonist suspension was poured into each pot. After seven days, seedlings were removed from the pots, the roots were washed with water, immersed in the pathogen inoculum suspension (OD_{540} = 0.2) for 1 min and replanted to the same pot.

For all modes of delivering antagonist and pathogen inoculation seedlings were kept in growth chamber at 30 °C ± 2, 12 h-photoperiod and observed daily for a week. Both disease incidence and severity were assessed. Disease severity was assessed using a scale that varied from 0 to 2, in which 0 = healthy plants, 1 = partially wilted plants (or when one or more leaves were wilted but turgid leaves could be observed) and 2 = all leaves were wilted.

The experiment was set in a randomized complete block design with 299 treatments (298 antagonists and 1 control). The control consisted of the immersion of seeds and/or of the root system in non-sterilized running tap water. Three replicates (blocks) were used for each treatment. Each evaluation method was considered an independent assay and they were not compared among each other. An experimental unit was one seedling per pot. Data were interpreted using descriptive analysis. The average disease intensity was calculated from the three replicates, in each evaluation method isolates that resulted in the smallest disease intensity were selected as potential biocontrol agents.

2.3. Control of eucalyptus bacterial wilt

The most promising strains and a commercial mixture of biological control agents, Rizolyptus®, were evaluated for bacterial wilt control. Rizolyptus® (Biosoja, São Joaquim da Barra, SP, Brazil) is a liquid inoculant formulated with three strains of *Bacillus subtilis* (10^8 CFU/mL). Seeds of the Urograndis hybrid (AEC I144) were seeded in 128-cell polyethylene trays, containing Plantmax and kept for 50 days in a greenhouse with controlled temperature (26 ± 2 °C). Plants had their roots washed in water and immersed for 1 min in the antagonist suspension (OD_{540} = 0.2). In addition, 25 mL of the propagule suspension of each of the antagonists was distributed in each pot and seedlings were transplanted to the soil mixture. After seven days, plants were removed from pots and the roots were washed and immersed in the inoculum suspension of *R. solanacearum* RS 295 (OD_{540} = 0.2) for 1 min and re-transplanted. After inoculation, the plants were kept in growth chamber at 30 ± 2 °C, 12 h-photoperiod, for up to 40 days. Two control treatments were used: immersion in water and then inoculation with the pathogen and immersion in water and uninoculated. Wilt incidence was assessed at every 5 days, from the fifth to the 25th day or 40th day after inoculation in the first and second/third replication, respectively. For disease confirmation, random samples were taken from wilted plants and an exsudation test was conducted (Alfenas et al., 2006).

Disease progress curves with values of wilt incidence (percentage of wilted plants) were constructed. The area under the disease progress curve was calculated for each plot according to the method of Shaner and Finney (1977), using time in days after symptoms appearance.

All treatments were assessed at three different times, thus comprising replication in time (blocks). At each time 10 pots with one plant were used for each treatment (pseudoreplicates). The first batch of treatments was assessed in 50-day-old eucalyptus seedlings, while the second and third were performed using 90-day-old seedlings.

The first experiment was set in randomized complete block design (RCBD), with 11 treatments (nine antagonists and two controls) applied to 10 pseudoreplicates and replicated in three different occasions (blocked on time). The second and third experiments were also set in RCBD, but had 8 treatments: the three best antagonists from the first experiment (UFV-11, UFV-56 e UFV-62), one antagonist with the worst result (UFV-32), one of intermediate performance (UFV-229), Rizolyptus, and the two controls. After checking for normality and homoscedasticity, analysis of variance (ANOVA) and mean comparisons by Fisher's Protected Least Significant Difference (LSD) test, both at $\alpha = 0.05$, were performed. All statistical analyses were done with the R Package (R Core Team, 2013).

2.4. Identification of antagonists

The genomic DNA of selected antagonists was extracted using Promega Genomic Wizard DNA Purification kit (Promega, Madison, WI, USA). A partial sequence of the 16S rRNA gene was amplified by PCR with the following primer pair: E8F-AGA GTTT GAT CCT GGC TCAG and 1115R-AGG GTT GCG CTC GTT G (Baker et al., 2003). The amplification cycles had a step of 94 °C for 2 min, followed by 30 cycles composed of denaturation at 94 °C for 30 s, primer annealing at 50 °C for 30 s and an extension step at 72 °C for 45 s. Final extension was achieved at 72 °C for 10 min. The PCR product was sequenced in a MEGABACE sequencer at the Laboratório de Genômica do Instituto de Biotecnologia Aplicada à Agropecuária (BIOAGRO) of the Universidade Federal de Viçosa. The sequences were submitted to comparative analysis with the NCBI database (BLAST – Nucleotide Sequence Database).

The most promising isolates were identified at the species level by determining the profile of fatty acid methyl ester by gas chromatography (GC-FAME) and using The Sherlock® Microbial Identification System and comparisons with the ITSA library.

2.5. Colonization ability

The root system colonization capacity of selected isolates was evaluated as previously described (Silva and Romeiro, 2003). Shortly, seeds were disinfested by immersion in ethanol (70%) for 30s, and then, in NaClO (2%) for 3 min followed by washing with

sterilized water. Disinfested seeds were immersed in a suspension of rhizobacteria propagules for 12 h and then put to germinate in tubes containing Phytagel (Sigma) at 0.05%. Each rhizobacterium was tested in three replicates, and a replicate was considered as one seed in a tube. Root colonization was inferred based on the opacity of the Phytagel near the roots.

2.6. Investigation of the mechanisms of antagonism of the selected biocontrol agents

The production of water soluble antimicrobial substances was studied by double layer tests (Romeiro, 2007). The biocontrol agents were transferred to 523 agar medium in four equidistant points, and incubated for 48 h at 28 °C. After colony growth, plates were inverted and 1 mL of chloroform was placed in the inner part of each dish cover. After 20 min, the antagonists were killed and the plates were opened to eliminate chloroform residues. Five mL of semi solid medium with 0.1 mL of cell suspension of *R. solanacearum* were added to each plate to form an extra ~1 mm-thick layer. The plates were incubated again as previously described and examined for the presence/absence of inhibition halo after five days.

The inhibition by unspecific volatile antimicrobial compounds was studied using the inverted plate method (Romeiro, 2007). Aliquots of diluted suspensions of antagonist propagules (10^{-1}–10^{-8}) and of pathogen cells with $OD_{540} = 0.2$ were streaked onto 523 medium in Petri dishes. The dishes containing antagonist propagules dilutions were paired with the dishes containing the pathogen cells for 96 h at 28 °C. After 48 and 72 h, the number of colonies was recorded and the order of colony appearance was registered. Additionally, the size of *R. solanacearum* colonies was observed on a daily basis. The results were compared using the "*t*" test at $\alpha = 0.05$.

To check for siderophore production, the microorganisms were cultivated in King B medium for 48 h under agitation (Schwyn and Neilands, 1987). As control, each culture was grown in the same culture medium supplemented with Fe^{3+} 2 μM sterilized by filtration of $FeSO_4 \cdot 7\ H_2O$. Flasks were kept at 28 °C for 48 h and the medium with the bacterial growth was centrifuged and the precipitate disposed. Equal volumes (500 μL) of the supernatant and chrome blue S dye solution (indicator) were mixed in a test-tube. The indicator solution consisted of a mixture of 600 μL of hexadecyltrimethylammonium (HDTMA) at 10 mM and 3 mL of distilled water. An aliquot of 150 μL of ferric solution from $FeCl_3 \times 6\ H_2O$ 1 mM dissolved in HCl 0.01 N was added to each tube under agitation. An additional aliquot of 750 μL of chrome blue S 2 mM and 0.4307 g of piperazine anhydrous was added to each tube (Schwyn and Neilands, 1987). The volume was completed to 10 mL with distilled water.

Test tubes containing the mixture were assessed within a 15 min-period and color change from blue to "reddish" indicated the production of siderophores by the antagonist. All glassware used in this assay were immersed on sulfochromic solution for 48 h and then washed several times in distilled water before used.

The production of HCN in TSA semi-solid culture medium (Trypticase soy agar 40 g L^{-1}) was also assessed (Guilbault and Kramer, 1966; Keel et al., 1989). Spores of actinomycetes and bacterial cells were dispensed into cavities (1 cm diameter) formed in partially melted TSA. Nylon screens were used to cover the culture medium. Filter papers were soaked in 5 mg of 4,4′-Dimethylene (-N, N-Dimethylaniline) solution and 5 mg of Ethyl acetoacetate dissolved in 2 mL of chloroform. The filter paper was then put on top of the nylon screen. The plates were put in plastic boxes (11 cm length × 11 cm width × 3 cm height) for 72 h at 28 °C. The appearance of a brown colored stain in the filter paper indicates HCN production by the antagonist.

The possibility of ammonium production was studied by seeding the antagonist bacteria in test tubes containing liquid media. A strip of litmus paper was placed between the border of the tube and the cotton plug used as cap. The color change from purple to "bluish" in three days indicated ammonium production by the bacteria.

3. Results

3.1. Isolation

A total of 298 microorganisms associated to the rhizosphere and roots of eucalyptus were isolated: 98 colonies of actinomycetes and 200 of bacteria of different morphology, height, color and shape were selected. The isolates were coded and stored at the Laboratório de Bacteriologia of the Departamento de Fitopatologia at Universidade Federal de Viçosa, Minas Gerais state, Brazil.

3.2. Mass selection

When roots were immersed in both antagonist and pathogen suspensions (RASAS), the most effective strains in reducing bacterial wilt intensity were UFV-11, 40 and 56 (Fig. 1A). When rhizobacteria were applied by the immersion of seeds in the suspension of antagonist propagules and then in the pathogen inoculum suspension, seven strains, UFV-32, 56, 62, 101, 170, 229 and 270, protected the seedlings from infection (Fig. 1B). High variability and inconsistent results were observed for the MSIS application method, therefore potential biocontrol agents were selected based on the results of the RASAS and MSRI tests (Fig. 1C).

The threshold of 33.33% incidence (1 out 3 plants wilted) was established for comparing the different antagonist delivering procedures. For MSRI, RASAS, and MSIS 13, 18, and 39 strains allowed wilt to develop in one of the three plants of the experimental unit (33.33% incidence).

The selected strains were UFV-11, 32, 40, 56, 62, 101, 170, 229 and 270. The strain UFV-56 was the only one capable of protecting tomato plants in all application methods.

3.3. Control of eucalyptus bacterial wilt

In the first experiment isolates UFV-56 and UFV-62 were more effective in controlling eucalyptus bacterial wilt and significantly reduced disease incidence ($P < 0.001$ for both isolates) compared to the control. The remaining treatments did not differ from the control. Isolate UFV-62 was capable of fully suppressing bacterial wilt in two replicates (Fig. 2A). In the second experiment, disease incidence was significantly reduced by UFV-56 ($P < 0.001$), 62 ($P = 0.029$) and 229 ($P = 0.032$), and the commercial product Rizolyptus ($P < 0.001$). Isolate UFV-56 and Rizolyptus resulted in the smallest values of the area under the disease progress curve (AUDPC) (Fig. 2B). In the third experiment, only plants treated with isolate UFV-56 had significantly lower ($P < 0.001$) values of AUDPC than the control (Fig. 2C).

3.4. Root colonization

Isolate UFV-11 was not capable of colonizing the roots of tomato seedlings in any of the three replicates. Isolates UFV-32 and UFV-101 did not colonize eucalyptus roots. All other strains formed an opaque zone, of bigger density, adjacent to the roots, which indicates the root colonization by the bacteria.

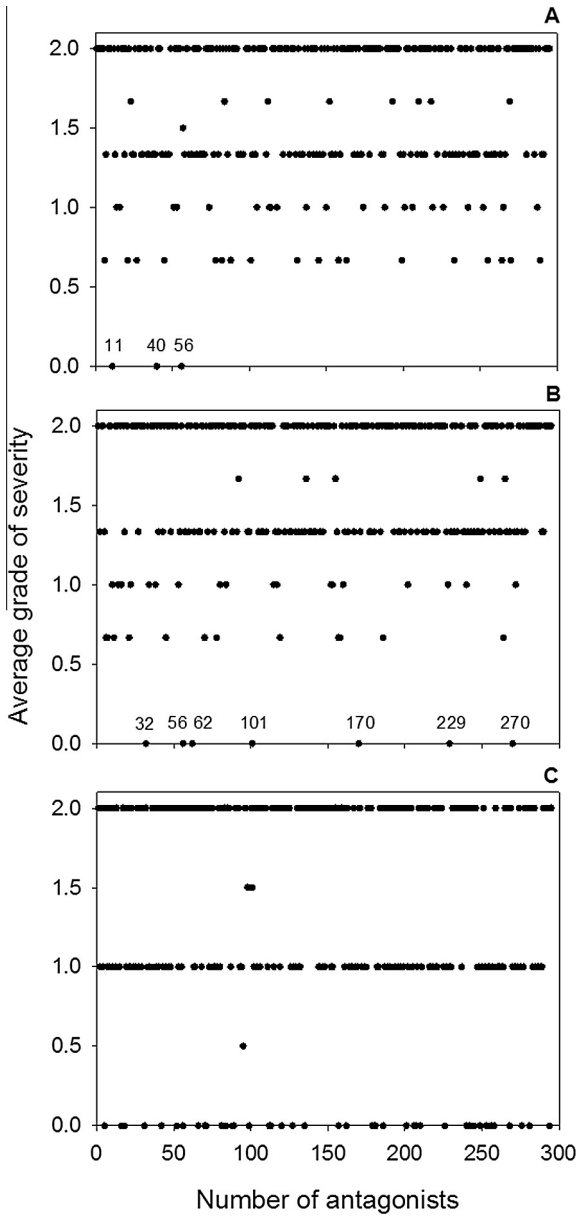

Fig. 1. Scatter plot of the average severity values (average of three replicates) of bacterial wilt caused by *Ralstonia solanacearum* in tomato plants treated with rhizobacteria in three application methods: (A) immersion of roots in the suspension of rhizobacteria and then in inoculum suspension of pathogen (RASAS); (B) microbiolized seeds followed by the immersion of roots in inoculum suspension (MSRI) and (C) microbiolized seeds and transplanting to soil infested with *R. solanacearum* (MSIS).

3.5. Strain identification

Analysis of the 16S rRNA sequences revealed that strains UFV-32 and UFV-40 belong to the *Pseudomonas* genus, UFV-101 to the genus *Stenotrophomonas*, and strains UFV-11, UFV-56, UFV-62, UFV-170, UFV-229, UFV-270 to the genus *Bacillus*. The GC-FAME analysis identified UFV-56 as *Bacillus thuringiensis* (SIM = 0.739) and UFV-62 as *Bacillus cereus* (SIM = 0.815).

3.6. Laboratory assays

No strains produced low molecular weight, water soluble substances capable of inhibiting *R. solanacearum*. Only strains UFV-62 and UFV-229 produced siderophores (Table 1). Five out of nine strains tested, UFV-11, 40, 56, 101, and 229, produced HCN. Ammonium was produced by UFV-40, 101, 229 and 270 strains, being more evident for the first two (Table 1).

Volatile compounds produced by the antagonists did not reduce the number of colonies of R. solanacearum at the 10^{-4}–10^{-8} dilutions after 48 and 72 h. However, the *B. thuringiensis* (UFV-56) produced some kind of volatile compound capable of delaying in two days the growth of *R. solanacearum* at the 10^{-2}–10^{-4} dilutions and of reducing colony size in all dilutions. At the 10^{-1} dilution, the *B. thuringiensis* isolate completely inhibited pathogen growth.

4. Discussion

Achieving satisfactory levels of bacterial wilt control by means of biological control agents (BCA) is a challenging task due to the difficulties in finding good BCAs and because of the highly aggressive nature of the disease. The strategy for efficient antagonist selection should provide opportunity for the BCA to affect the host-pathogen interaction (Pang et al., 2009) where it takes place in the plant. In addition to efficacy, BCAs should preferably come from similar environment to those found where they are intended to be used (Pliego et al., 2011). The strategies used in the present work allowed for relatively high numbers of bacteria and actinomycetes isolates (~300 isolates) obtained from eucalyptus and tomato plants grown in soils and environmental conditions similar to those where antagonists will be used. We anticipate only minor difficulties regarding the capacity of thriving and persistence of the selected BCAs strains in soils in eucalyptus growing area in Brazil.

Whole plant tests are usually referred to as the most convincing strategy to select BCAs (Daayf et al., 2003). Even though selection of BCAs commonly takes place using the host species of interest, the utilization of a model-system based on tomato plants was successful and allowed the selection of agents capable of reducing the intensity of bacterial wilt in eucalyptus. Similar result was reported by Gopalakrishnan et al. (2011) who selected seven rhizobacteria strains from the rhizosphere of rice plants which apparently multiplied in and colonized sorghum roots. However, when model-systems are used, the selection may not be as efficient because of the risks of not taking into consideration important events of the plant-pathogen-antagonist interaction. Nevertheless, in this study, the selection based on trials using tomato plants resulted in efficient BCAs for eucalyptus bacterial wilt. Bioassays with tomato plants are advantageous because of the higher susceptibility of this host and rapid disease development.

Two strategies can be planned when seeking effective biological control: preventive and curative (Usall et al., 2008). The preventive strategy is aimed at protecting plants against pathogen for a long time and the curative is usually applied to suppress the disease for a limited period of time (Maloy, 2005). The mode of action of the BCAs is apparently influenced by the delivery methods used in the selection stage (Knudsen et al., 1997). A complex selection method involving the antagonist, pathogen and plants is advocated because it can closely mimic the field conditions. On the other hand, it is more difficult to gather detailed information on the mode of action (Pliego et al., 2011). In this study, we believe that BCAs selected after using MSRI (UFV-32, 56, 62, 101, 170, 229 and 270) reduced the incidence of bacterial wilt by inducing plant resistance while those selected by RASAS (UFV-11, 40 and 56) most likely act by direct antagonism because the pathogen and the antagonist could be found concomitantly in the same substrate.

The modes of action of rhizobacterial isolates effective in suppressing bacterial wilt in tomato were studied. Colonization of plant roots is the first step when considering the interaction of both pathogenic and beneficial rhizobacteria with plants. The colonization of roots by BCAs before pathogen establishment

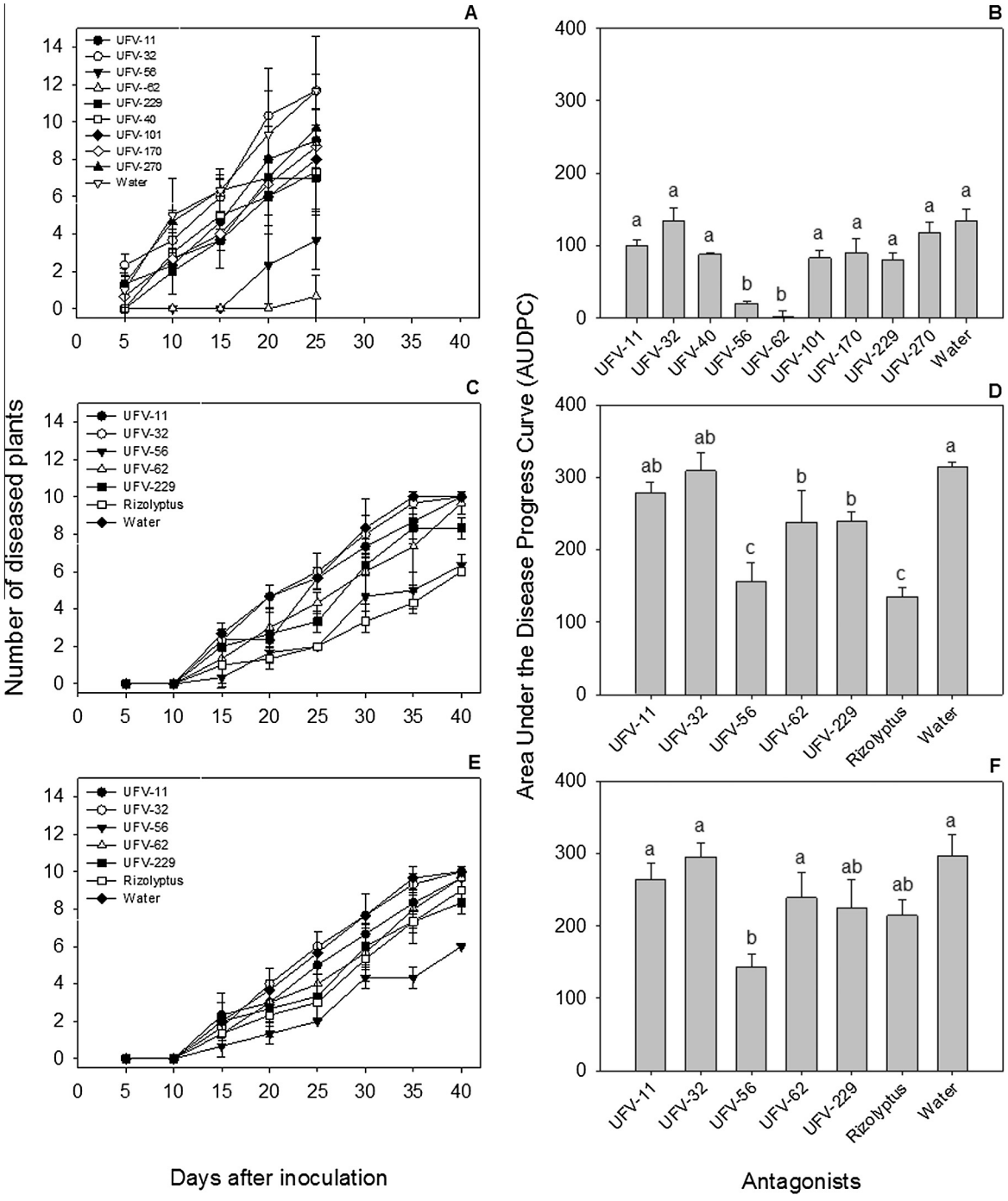

Fig. 2. Progress of bacterial wilt (*Ralstonia solanacearum*) in eucalyptus plants treated with rhizobacteria with the respectively AUDPC (% plant wilt-day). (A) and (B) Experiment 1; (C) and (D) Experiment 2; (E) and (F) Experiment 3. The numbers in front of each line refers to the UFV strain code.

favors biological control by preventing pathogen penetration (Haggag and Timmusk, 2008). Additionally, fast colonization of roots are also important factors for the establishment and introduction of BCAs in the rhizosphere and biocontrol efficacy (Gamalero et al., 2003; Kloepper and Beauchamp, 1992). Among the antagonists selected based on the tomato bioassays, only UFV-11 strain was not capable to colonize tomato roots, but it was capable of controlling the disease in this host. This is probably due to its incapacity of adhering to tomato seeds and/or of colonizing tomato roots. UFV-11 was able to colonize eucalyptus roots and we speculate that growing in the surface could have prevented penetration and infection. Gotz et al. (2006) reported that soilless roots were more efficiently colonized by *Pseudomonas putida* (PRD16) and *Enterobacter cowanii* (PRF116) when compared to microbiolized seeds. Strain UFV-11 may have colonized tomato roots less intensively when applied by seed microbiolization than when carried by contact with soilless roots. The sparse or no-colonization of tomato seeds can explain the lack of disease suppression, given that the strain presented itself as a potential antagonist when assessed using the RASAS method, but was not effective when delivered using MSIS or MSRI.

The UFV-32 and 101 strains controlled bacterial wilt in tomato plantlets, but did not prevent disease development in eucalyptus. The non-reproducibility of results in eucalyptus is putatively

Table 1
Capacity of root system colonization, ammonium, siderophores and hydrogen cyanide (HCN) production and direct antibiosis by selected antagonists as potential agents for the bacterial wilt (*Ralstonia solanacearum*) biocontrol.

Tested antagonists	Root colonization		Ammonium	Antibiosis	Siderophore	HCN	Volatiles
	Tomato	Eucalyptus					
UFV-11	–	+	–	–	–	+	–
UFV-32	+	–	–	–	–	–	–
UFV-40	+	+	+	–	–	+	–
UFV-56	+	+	–	–	–	+	+
UFV-62	+	+	–	–	+	–	–
UFV-101	+	–	+	–	–	+	–
UFV-170	+	+	–	–	–	–	–
UFV-229	+	+	+	–	+	+	–
UFV-270	+	+	+	–	–	–	–

– = Negative result.
+ = Positive result.

associated with the failure in colonizing eucalyptus roots due to quantitative and/or qualitative differences in substances secreted by the hosts. It also should be highlighted that the inoculum concentration of the antagonist suspensions were standardized for the eucalyptus bioassay, but no standardization was done for the treatment of tomato roots. In some cases, the antagonist performance was demonstrated to be density-dependent (Silva et al., 2000), thus variations in BCA concentration could have been a source of variation in the assays. Based on the *in vitro* root colonization test, some strains capable of colonizing tomato roots did not colonize eucalyptus roots. The amount and composition of the exsudates secreted by the roots, like sugars and amino acids, can interfere in the colonization process, the size of the BCA population and the activity of rhizobacteria (Buchenauer, 1998). In addition to the BCA population effects, environmental factors, particularly temperature, may have affected the colonization of the root system by rhizobacteria, as previously reported in other systems (Stirling, 1981; Hatz and Dickson, 1992). In our study, the root system of tomato plants was treated with BCAs in May, when temperatures were higher than in June, when the strains were tested in the eucalyptus root system.

The *B. thuringiensis* (UFV-56) and *B. cereus* (UFV-62) should be carefully evaluated for their high potential as BCA for tomato bacterial wilt. UFV-56 was the only antagonist able to quickly adapt to tomato/eucalyptus and maintain a high population in the tomato rhizosphere for a long time. Currently, eucalyptus is propagated mostly by rooting young and tender twigs in nurseries. Outbreaks of eucalyptus bacterial wilt are commonly related to planting infected seedlings (Alfenas et al., 2006). Coating seeds with antagonists and supplementing the substrate with biocontrol agent propagules can be a delivery strategy. However, besides being capable of multiplying and surviving in the rhizosphere and in the rhizoplane, isolates need to be able to compete and to adapt in a species-rich soil environment (Lugtenberg et al., 2001). The *B. thuringiensis* strain, when in high population, produced volatile compounds which inhibited the growth and multiplication of *R. solanacearum*. However, it is not possible to safely conclude about the nature of these substances. This strain does not produce ammonium, but it produces hydrogen cyanide (HCN) which can inhibit pathogen growth. This volatile compound was capable of delaying in two days the growth of *R. solanacearum* at the 10^{-2}–10^{-4} dilutions and the toxic effect completely inhibited pathogen growth at the 10^{-1} dilution. Other isolates (UFV-11, 40, 101 and 229) produced HCN, but the amount was not sufficient to restrict the growth of *R. solanacearum* as efficient as UFV-56. Nevertheless, production of HCN does not seem to be the only responsible variable for disease control by UFV-56. It is postulated that the control efficiency was also due to the reduction of the pathogen activity on the rhizosphere and by the activation of the plant defense systems and efficient colonization of rhizobacteria preventing contact of the pathogen with the plant root.

It is highly likely that isolate *B. cereus* (UFV-62) controlled bacterial wilt mainly due to siderophore production. Many biocontrol agents act by this mechanism (Ran et al., 2005a). Nevertheless, there was no correlation between *in vitro* siderophore production by *P. putida* WCS358r, *P. fluorescens* WCS374r and WCS417r and *Pseudomonas aeruginosa* 7NSK2 and the control of eucalyptus bacterial wilt (Ran et al., 2005a). Although not properly characterized, the siderophores produced by the *B. cereus* UFV-62 may be of the catecholate or hydroxamate type, but more detailed experiments designed to address this issue need to be performed in the future.

Some bacteria, in addition to protecting the plant, have the potential to promote plant growth (Compant et al., 2005). In the present work, plant growth-promoting rhizobacteria decreased the incidence of bacterial wilt of eucalyptus, but the mechanisms behind this process were not studied. So far BCAs used for the control of bacterial wilt include avirulent mutants of *R. solanacearum* (Trigalet and Trigalet-Demery, 1990); mycorrhizae (Zhu and Yao, 2004); endophytic bacteria (Ji et al., 2008), fungi (Masunaka et al., 2009), congeneric rhizobacterium (Wei et al., 2013) and a few antagonistic rhizobacteria as *Pseudomonas* spp. (Ran et al., 2005a,b), *Stenotrophomonas* spp. (Messiha et al., 2007), *Serratia* spp. (Guo et al., 2004), *Acinetobacter* spp. and *Enterobacter* spp. (Xue et al., 2009). In the present work, *B. thuringiensis* and *B. cereus* were selected as potential BCA. These are interesting organisms for developing BCA formulation because they are capable of forming endospore and resist under adverse environmental conditions (Raaijmakers et al., 2002). Isolates of both species were reported as efficient biocontrol agents against *R. solanacearum* (Hyakumachi et al., 2013).

In summary, two native isolates (UFV-56 and UFV-62) and the commercial product Rizolyptus decreased the incidence of bacterial wilt in eucalyptus. The *B. thuringiensis* UFV-56 and the *B. cereus* UFV-62 are likely to adapt to agrosystems where eucalyptus and tomato plants are cultivated. Most likely, the *B. thuringiensis* isolate has a potential to be used as bio-bacteriocide under field conditions. Future studies need to be carried out testing organisms in mixtures, using different concentrations of the BCAs to increase the efficacy of biological control and applicability in other hosts of *R. solanacearum*.

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